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Exosomes contribute to the transmission of anti-HIV activity from TLR3-activated brain microvascular endothelial cells to macrophages

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Abstract

Human brain microvascular endothelial cells (HBMECs), the major cell type in the blood-brain barrier (BBB), play a key role in maintaining brain homeostasis. However, their role in the BBB innate immunity against HIV invasion of the central nervous system (CNS) remains to be determined. Our early work showed that TLR3 signaling of HBMECs could produce the antiviral factors that inhibit HIV replication in macrophages. The present study examined whether exosomes from TLR3-activated HBMECs mediate the intercellular transfer of antiviral factors to macrophages. Primary human macrophages could take up exosomes from TLR3-activated HBMECs. HBMECs-derived exosomes contained multiple antiviral factors, including several key IFN-stimulated genes (ISGs; ISG15, ISG56, and Mx2) at mRNA and protein levels. The depletion of exosomes from TLR3-activated HBMECs culture supernatant diminished HBMECs-mediated anti-HIV activity in macrophages. In conclusion, we demonstrate that exosomes shed by HBMECs are able to transport the antiviral molecules to macrophages. This finding suggests the possibility that HIV nonpermissive BBB cells (HBMECs) can help to restore the antiviral state in HIV-infected macrophages, which may be a defense mechanism against HIV neuroinvasion.

Keywords

HBMECs; BBB; TLR3; Exosomes; Antiviral

The blood-brain barrier (BBB) consists mainly of human brain microvascular endothelial cells (HBMECs), astrocytes and pericytes. As a major cell population in BBB, HBMECs

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Authorship contributions

JL and WH conceived the project and designed the experiments; JL, SL, XW, YZ, and RZ performed the experiments; JL and WH analyzed the data.

Disclosure of conflicts of interest

The authors declare that there is no conflict of interest.

have a crucial role in maintaining brain homeostasis. In addition, HBMECs are involved in the immunological processes of the BBB, including cytokine-mediated inflammation and regulation of the brain influx of immune cells (Abbott et al., 2006; Lampron et al., 2013). Toll-like receptor 3/retinoic acid inducible gene I (TLR3/RIG-I) are two sets of pathogen-recognition receptors (PRRs) to sense the genetic materials of viruses. During the HIV life cycle, HIV provides ample pathogen-associated molecular patterns (PAMPs) that can be detected by a variety of PRRs of the innate immune system (Altfeld and Gale, 2015; Lee, 2013; Mogensen et al., 2010). Among the PRRs, TLR3/RIG-I have been implicated in sensing dsRNA structures during HIV infection (Berg et al., 2012; Mogensen et al., 2010). In addition, the HIV lentiviral vector immunogenicity has been associated with TLR3-mediated dendritic cell activation (Breckpot et al., 2010). We recently showed that the HBMECs possess functional TLR3/RIG-I signaling pathways, the activation of which could induce antiviral factors that suppressed HIV replication in macrophages (Li et al., 2013b). However, it is unclear about the intercellular communication between HBMECs and macrophages in the context of immunity against HIV. Recent studies have shown that exosomes play an important role in intercellular communications by acting as a carrier for essential cell-specific information to target cells (Bang and Thum, 2012; Camussi et al., 2010; Smalheiser, 2009). Exosomes contributed to the cell-to-cell transmission of antiviral activity from liver nonparenchymal cells (LNPCs) to hepatocytes against hepatitis viruses (Giugliano et al., 2015; Li et al., 2013a). We thus examined the role of exosomes in HBMECs-mediated antiviral activities against HIV. We also investigated the mechanism(s) of the exosome actions.

As a physiologically relevant to *in vivo* BBB system, HBMECs have been widely used as an *in vitro* model of BBB (Naik and Cucullo, 2012). The cells were first stimulated with the dsRNA mimic PolyI:C (high-molecular weight) for 6 h. The cultures were then washed and replenished with fresh exosome-free medium. Culture media were collected at 24, 48 and 72 h post-stimulation. Exosomes were isolated by the isolation reagent (Invitrogen, Grand Island, NY) and dissolved in sterile phosphate buffered saline (PBS) or radio-immunoprecipitation assay lysis (RIPA) buffer supplemented with protease inhibitor cocktail. Purified exosomes were then characterized by an electron microscopy (ETEC U-I, Perkin Elmer, Waltham, MA). Scanning electron microscopy analysis of the purified pellets identified small membrane vesicles of ~100 nm in diameter, which have a typical cup-shaped exosome structure (Fig. 1A). The concentrations of the RIPA exosome lysates were determined by the Chemcruz BCA Protein Assay kit (Santa Cruz, Dallas, TX) and equal amount of exosomal and cellular lysates (20 µg) were resolved on 4–12% Bis-Tris NuPAGE gels. Western blotting analysis showed the presence of exosomal markers (Alix, CD63, TSG101), as well as several conserved endothelial exosomal proteins, including PECAM-1, LAMP-2, and CD81 (Fig. 1B) in exosome lysates. The CD63 exhibited in variably glycosylated forms in cells but was in the most extensively glycosylated form in exosomes (Fig. 1B). The lack of EEA1 (an endosomal marker), cytochrome c (a mitochondrial marker) and actin (cytoskeleton marker) in the exosomal lysate confirmed the purity of the isolated exosomes. We examined whether macrophages could take up the exosomes derived from HBMECs. As shown in Fig. 1C, the PKH-67 labeled exosomes were internalized in macrophages as

evidenced by the green puncta in the macrophages with red PKH-26 staining of cell membrane (Fig. 1C).

We next examined exosome-mediated antiviral activity in macrophages. Monocytes (purity >95%) were obtained from the Center for AIDS Research of the University of Pennsylvania School of Medicine and differentiated into macrophages in 48-well plates (Corning CellBIND Surface, Corning Incorporated, Corning, NY) at 2.5×10^5 cells per well for 7 days. The cells were pretreated with exosomes (5 $\mu\text{g/mL}$) derived from unstimulated or PolyI:C-stimulated HBMECs for 24 h and then infected with HIV isolate Jago, a macrophage-tropic isolate derived from cell-free cerebral spinal fluid from a patient with confirmed HIV-associated dementia (Chen et al., 2002) (provided by Dr. Dennis Kolson, University of Pennsylvania). As we previously showed that PolyI:C could directly trigger antiviral response of macrophages (Zhou et al., 2010), we washed off the ligand after 6 h stimulation of HBMECs to exclude the possibility to contaminate PolyI:C in the exosome fractions. The exosomes isolated thereafter contained no PolyI:C as evidenced by the absence of red fluorescence when using rhodamine-conjugated PolyI:C for stimulation (data not shown). As shown in Fig. 1D, macrophages treated with the exosomes from PolyI:C-stimulated HBMECs had significantly lower levels of intracellular GAG mRNA and p24 expression than untreated macrophages. To validate the viral output data at replication level, we measured the extracellular HIV reverse transcriptase in the macrophage culture medium on the basis of the technique of Willey et al. (Willey et al., 1988) with modifications as described previously (Wang et al., 2009). Consistent with the viral inhibition at both intracellular mRNA and protein (Fig. 1D) levels, the HIV RT activity was significantly lower in macrophage cultures treated with exosomes from PolyI:C-activated HBMECs. To confirm the role of the exosomes in HBMECs-mediated anti-HIV activity, we next examined the antiviral potency of HBMECs supernatant with or without exosome depletion. To deplete exosomes, the culture supernatant from PolyI:C-stimulated HBMECs was incubated with anti-CD63 antibody-conjugated Dynabeads overnight at 4 °C and then separated in magnetic field. Fig. 1E shows that supernatant from PolyI:C-stimulated HBMECs significantly suppressed HIV replication in macrophages. However, the depletion of exosomes from HBMECs supernatant diminished HBMECs-mediated anti-HIV activity in macrophages (Fig. 1E).

We previously showed the TLR3 signaling of HBMECs by PolyI:C could induce IFN- λ (Li et al., 2013b). We therefore examined whether IFN- λ is involved in the exosome-mediated antiviral activity. However, we found that exosomes from TLR3-activated HBMECs contained much lower levels of IFN- λ than the HBMECs culture supernatant (Fig. 2A and B). We then determined whether the exosomes carry the antiviral executor IFN-stimulated genes (ISGs). Because the cycle threshold (Ct) values of some exosomal mRNA genes were above 35, which is not proper to use the comparative Ct method for quantitative analysis, the PCR amplification products of exosomal genes were resolved on 2% agarose gel and the band density was compared. As shown in Fig. 3A and B, PolyI:C stimulation induced the mRNA expression of several ISGs (ISG15, ISG56, OAS-1, Mx2) with well-known antiviral activity in both HBMECs and HBMECs-derived exosomes. The highest levels of the cellular ISGs were induced by the low dose of PolyI:C (0.01 $\mu\text{g/mL}$), while higher doses (1–10 $\mu\text{g/mL}$) of PolyI:C were needed to induce the observable levels of the ISG mRNAs in

exosomes (Fig. 3A). The peak induction of the cellular ISG mRNA was observed at 24 or 48 h post-stimulation (Fig. 3B). In contrast, there was gradual and steady increase of the ISGs in exosomes within 72 h post-stimulation (Fig. 3B). At protein levels, we also observed an increase in the antiviral ISGs (ISG15, ISG56, OAS-1, Mx2) in HBMECs upon PolyI:C stimulation, among which ISG15, ISG56 and Mx2 were enriched in the exosomes from PolyI:C-stimulated HBMECs (Fig. 3C and D). We further examined the antiviral signaling triggered by exosomes in macrophages. As shown in Fig. 4, treatment of macrophages with the exosomes isolated from PolyI:C-stimulated HBMECs induced the expression of the ISGs (ISG15, ISG56, Mx2) at both mRNA and protein levels in macrophages (Fig. 4A and B).

BBB not only serves a physical barrier but also participates in the CNS innate immunity against viral infections (Abbott et al., 2006). Several days after primary infection, HIV can be detected in the brain, indicative of early viral neuroinvasion (Gray et al., 1996). However, HIV-associated neurodegeneration is clinically manifested years after seroconversion and with late histopathological alterations of BBB (Gonzalez-Scarano and Martin-Garcia, 2005; Pereira and Nottet, 2000). The asynchronous occurrence of these events highlights the importance of BBB in controlling the HIV infection and maintaining the brain homeostasis. Recently, viral sensing at the BBB has become a focus of research on understanding the peculiar nature of the CNS immunology (Daniels and Klein, 2015). HIV infection, indeed, provides ample PAMPs for innate immune sensing (Iwasaki, 2012; Lee, 2013). Viral neuroinfection, release of coding or non-coding RNAs from the infected cells at the sites of brain injury or degeneration would require nucleic acid sensing pathways in the cells of neuronal and non-neuronal origin (Salmina et al., 2015). Although HBMECs are not permissive for HIV infection, there is still chance of viral nucleic acid presence inside cells during HIV neuroinvasion across the BBB (Argyris et al., 2003; Blank et al., 2016; Chaudhuri et al., 2008; Mukhtar et al., 2002). It has been reported that a variety of PPRs are evolved to sense the genetic materials of HIV, such as the archetypical PPRs TLRs (TLR3, TLR7/8, and TLR9) and RIG-I-like receptor (RLR) (Iwasaki, 2012). HBMECs have been revealed to possess functional TLR3/RIG-I signaling pathways (Li et al., 2013b). In this context, the TLR3/RIG-I activation of HBMECs should contribute to the protection of the CNS from HIV infection.

The activation of TLR3/RIG-I induces IFN- λ and inhibits HIV replication in macrophages (Li et al., 2013b). The involvement of TLR3 signaling was confirmed by either treatment of cells with Bafilomycin A1, a proton pump inhibitor that blocks the endosomal acidification (Li et al., 2013b), or with the specific TLR3/dsRNA complex inhibitor that can block the binding of dsRNA to TLR3, which both attenuated the antiviral potency. Although the pleiotropic effect of TLR3 signaling may activate NF- κ B pathway, leading to the production of inflammatory cytokines as well, it has been reported that there is minimal induction of inflammatory cytokines by the delivery of liposome-encapsulated PolyI:C inside cells, the means of stimulation used in our study (Dauletbaev et al., 2015). In addition, both TLR3 and RIG-I signaling pathways were contributable to the induction of antiviral IFN- λ from HBMECs (Li et al., 2013b). We here showed that the induced IFN- λ predominately presented freely in the culture supernatant (Fig. 2). This is consistent with the preceding study that an antibody to IFN- λ receptor 1 (IFNLR1) could compromise the antiviral

activity of the HBMECs culture supernatant. We noticed that the stimulatory effect of exosomes from TLR3-activated HBMECs cultures on levels of ISGs in primary macrophages was much weaker than that of direct PolyI:C treatment of macrophages (Fig. 4B). However, it has been reported the innate immunity of macrophages was compromised in the context of HIV infection as viral evasion strategies (Mogensen et al., 2010; Solis et al., 2011; Tomlinson et al., 2014). It is thus of significance that the BBB provides complementary antiviral activity from bystander cells to the HIV-infected macrophages during HIV neuroinvasion. A recent study (Lazear et al., 2015) showed the TLR3-inducible IFN- λ in HBMECs could protect the mouse neuronal cells from West Nile virus infection by reducing transport of large molecules across the BBB. Evidently, TLR3 signaling of HBMECs has a crucial role in the CNS protection from viral infections.

As membrane vesicles, exosomes are crucial in the intercellular communications and innate immune regulation. It was reported that HBV infection resistant liver resident cells such as Kupffer cells and liver sinusoidal endothelial cells (LSECs) are able to transmit antiviral activity to HBV-susceptible hepatocytes (Li et al., 2013a). The presence of a variety of antiviral molecules, particularly APO-BEC3G (A3G), in the exosomes enables an effective arsenal of liver immunity against HBV in the context of IFN- α -based clinical therapy. However, in brain endothelial cells, A3G expression was expressed at a very low level and there was no induction by TLR3 stimulation of HBMECs (Fig. 3A and B). This is in agreement with our previous study that TLR3 activation has little effect to induce IFN- α of HBMECs. Given the active interaction between pathogen and endothelial cells at the blood-vasculature interface, it is conceivable that other types of endothelial cells or epithelial cells could also mount a similar innate immune response (Berrich et al., 2012; Cines et al., 1998). Our earlier studies also showed that the human lung-derived microvascular endothelial cells (HMVEC-L) could recapitulate the IFN- λ induction after TLR3 activation (Li et al., 2013b), which might contribute to the protection of blood–air barrier (alveolar–capillary barrier). A recent study (Giugliano et al., 2015) demonstrated that human liver endothelial cells could also mount HCV PAMP-mediated activation and release ISG-enriched exosomes, which inhibited HCV replication of liver parenchymal hepatocytes. These data and findings, collectively, have introduced and expanded the idea that vascular endothelial cells lining the entire circulatory system, from liver to brain capillaries, may be a viral “firewall” or act as active “defendothelium” in sensing and controlling viral infection (Klenerman and Ramamurthy, 2015).

Collectively, we have presented evidence that TLR3 activation-induced antiviral factors in HBMECs could be transferred to macrophages through the release and internalization of exosomes. Because the viruses such as HIV have evolved mechanisms to evade TLR3/IFN-mediated intracellular innate immunity in the target cells (Finlay and McFadden, 2006; Guha and Ayyavoo, 2013), antiviral support from uninfected bystander cells is helpful in restoring antiviral state in the infected cells. Therefore, the delivery of multiple antiviral factors by exosomes may be an alternative strategy for treatment of HIV disease.

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Fig. 1. Characterization of exosomes of TLR3-stimulated HBMECs and the antiviral activity against HIV replication in macrophages

(A) Exosomes isolated from PolyI:C-stimulated HBMECs were examined by scanning electron microscopy. (B) The expression of exosomal markers (Alix, CD63, TSG101), and several conserved endothelial exosomal proteins (PECAM-1, LAMP-2, CD81), and EEA1 (an endosomal marker), cytochrome c (a mitochondrial marker) and Actin (cytoskeleton marker) was determined by western blotting. (C) The uptake of HBMECs exosomes labeled with PKH67 by macrophages. Macrophages were treated with PKH67-labeled HBMECs exosomes (green) for 18 h and then stained with PKH26 for general cell membrane labeling (red). Magnification: $\times 200$. (D) The inhibition of HIV replication by exosomes from PolyI:C-stimulated HBMECs. The intracellular HIV Gag mRNA and p24 protein levels and extracellular HIV reverse transcriptase activity were measured by qRT-PCR, Western blotting and RT assay, respectively. (E) The inhibition of HIV replication by HBMECs culture supernatant with or without exosome depletion. Data were obtained from at least three times of independent experiments and expressed as the mean $\pm$ standard deviation. *, $p < 0.05$; **, $p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. IFN-λ expression in the culture of HBMECs upon TLR3 stimulation

(A) IFN-λ concentration in the culture supernatant (SN) and cells-derived exosomes from HBMECs stimulated with indicated doses of PolyI:C (=P) for 48 h. (B) The IFN-λ contents in the different compartments of HBMECs culture. The IFN-λ were measured by ELISA. Data were obtained from three independent experiments and expressed as the mean $\pm$ standard deviation. *, $p < 0.05$; **, $p < 0.01$.

Fig. 3. Characterization of the antiviral factors in the exosomes of TLR3-stimulated HBMECs (A, B) The dose- (A) and time- (B) manners of intracellular and exosomal induction of the ISG mRNAs by PolyI:C of HBMECs. The cellular mRNAs were examined by qRT-PCR, normalized with GAPDH and expressed as fold of control (defined as 1) using the 2^{-Ct} method. Exosomal ISG mRNA PCR products were resolved by agarose gel electrophoresis and visualized on a Bio-Rad Gel imaging system. (C, D) The dose- (C) and time- (D) manners of intracellular and exosomal induction of ISG proteins by PolyI:C of HBMECs determined by western blotting. Data were obtained from three independent experiments and expressed as the mean $\pm$ standard deviation. Representative images of agarose gel and blot from three independent experiments were shown. *, $p < 0.05$; **, $p < 0.01$.

Fig. 4. Induction of ISGs in macrophages by exosomes derived from TLR3-activated HBMECs culture

Primary human macrophages were treated with exosomes from PolyI:C (1 $\mu\text{g/mL}$) or vehicle (Lyo) stimulated HBMECs cultures. Cells directly treated with PolyI:C (1 $\mu\text{g/mL}$) were used as a positive control. The mRNA and protein levels of ISGs were examined by qRT-PCR and western blotting, respectively. Data were obtained from three independent experiments and expressed as the mean $\pm$ standard deviation. Representative images of blot from three independent experiments were shown. *, $p < 0.05$; **, $p < 0.01$.